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13. ABSTRACT (Maximum 200 Words)

We have been working since the 1980s, for the past 5 yrs under DOD support, on novel ways to resuscitate "unresuscitable" trauma victims. We focus on combat casualties who exsanguinate internally resulting within a few min in cardiac arrest (CA). We have conceived and documented the concept of "suspended animation (SA) for delayed resuscitation" using a hypothermic saline flush into the aorta after rapid (over 5 min) exsanguination (Ex) CA, using novel clinically relevant outcome models in dogs. With the use of saline flush we have achieved complete recovery after ExCA of up to 120 min at 7-10°C. This is the report on yr 6. In yr 6, we carried out studies to determine if SA could be effective in the setting of ExCA preceded by a prolonged period (1.5-2.5 h) of hemorrhagic shock. This scenario mimics the important situation where a casualty may be pinned down for a prolonged period of time prior to the arrival of either the medic or transport to a field hospital. To this end, we applied SA for 1 h after prolonged hemorrhage –which we produced for durations between 1.5 and 2.5 h. Prior to the induction of SA, the dogs were moribund with a marked metabolic acidosis. Nevertheless, SA was successful in achieving intact neurological outcome in this setting when it was followed by a 48 h period of mild hypothermia. This further supports the potential feasibility of SA in military and civilian ExCA. In Yr 6, we also developed a full rat model of SA that included resuscitation using miniaturized cardiopulmonary bypass. With this new rat model, we began investigation of the effect of reperfusion on the rat brain proteome after a 30 min period of normothermic and deep hypothermic CA. These studies will also allow us to define key secondary injury targets during prolonged SA and reperfusion and ultimately screen novel pharmacological adjuncts to hypothermia. We also advised industries for novel smart catheter insertion and cooling devices that we will need to bring SA to patients, and tested several prototypes. Finally, we published several manuscripts based on this work.

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A. ABSTRACT

NOVEL RESUSCITATION FROM LETHAL HEMORRHAGE
Suspended Animation (SA) for Delayed Resuscitation
Keywords: Hemorrhagic shock, cardiopulmonary arrest, trauma, hypothermia, resuscitation, ischemia, proteomics, reperfusion, delayed neuronal death, combat casualty, terrorism, transport

This study concerns presently unresuscitable military and civilian trauma-induced hemorrhage to severe hemorrhagic shock (HS) and cardiac arrest (CA). We have shown that mild hypothermia (tympanic T, Tty 34°C) increases survival time and rate after HS in rats. Our suspended animation (SA) studies in dogs in years 1-5 (1998-2003) used rapid exsanguination over 5 min to CA. We documented that aortic flush with cold saline to Tty 10°C at the start of CA can preserve viability of the organism during up to 90 min CA without trauma (120 min with special preservation solutions), and 60 min CA with trauma. Fourteen drugs failed to approach the breakthrough effect of hypothermia. We reduced flush solution volume requirement by re-circulating diluted venous blood via a heat exchanger. In yr 5 (2003) we increased the preservation limit for traumatic CA from 60 min to 120 min with post-CA plasma exchange to mitigate or prevent the coagulopathy and multiple organ failure associated with trauma. For year 6 (2004), we proposed two major lines of investigation. First we proposed to explore *in dogs* with trauma, slow continuous, controlled exsanguination over prolonged periods (1.5-2.5 h) to CA, to determine if SA (60 min or longer) could still be effective in preserving the organism for transport and delayed resuscitation –even if the organism was in shock for an extended period prior to arrest. This scenario mimics the situation observed in the Mogadishu conflict (as depicted in "Black Hawk Down") to maximize preservation time for transport and repair, under special analgesia. Remarkably, we were able to achieve successful SA of 1 h in dogs with profound metabolic acidosis, and marked elevations of lactate and potassium levels in serum. The use of conventional SA followed by 48 h of mild (34°C) systemic hypothermia during ICU care resulted in survival with intact neurological outcome in most dogs in this series. The remarkable advantage of SA over conventional CPR in preservation was also convincingly shown. Second, in a parallel set of experiments, we developed a full rat model of SA that included resuscitation using miniaturized cardiopulmonary bypass. With this new rat model, we began investigation of the effect of reperfusion on the rat brain proteome (using proteomics as assessed by 1D and 2D gel electrophoresis of the rat hippocampus) after a 30 min period of normothermic and deep hypothermic CA. Our initial results with this new model suggest that there is little obvious protein degradation during SA, however, reperfusion after a normothermic 30 min ischemic insult results in obvious protein degradation. These studies will also allow us to define key secondary injury targets during prolonged SA and reperfusion and ultimately screen novel pharmacological adjuncts to hypothermia. We also advised industries for novel catheter insertion and cooling devices that we will need to bring SA to patients, and we tested several prototypes for our industrial consultants. Finally, we published a number of manuscripts and chapters and presented numerous abstracts based on this work.

PI: Patrick Kochanek, MD

ANNUAL RESEARCH REPORT FOR USAMRMC/TATRC
September 2003 – September 2004

NOVEL RESUSCITATION FROM LETHAL HEMORRHAGE
Suspended Animation (SA) for Delayed Resuscitation
Project Year 6

INTRODUCTION

This research report for 2003/04 concerns our US Army funded research project on "Novel resuscitation from severe hemorrhage, suspended animation (SA) for delayed resuscitation" (PI: Dr. Kochanek, Co-PI: Dr. Tisherman), project yr 6 (academic yr 2003/04, FY-03). The work carried out during yr 6 involved studies in 2 separate models: Study I) SA induced in our established dog model after an exsanguination cardiac arrest (ExCA) preceded by prolonged (1.5-2.5 h) of hemorrhagic shock (HS), and study II) establishment of a rat model of SA including resuscitation using miniaturized cardiopulmonary bypass (CPB). This model will allow us to study mechanisms of secondary damage including protein and lipid degradation (using proteomics and lipidomics, respectively) and facilitate the screening of novel therapies. Overall, for these studies, a total of 41 about 1 wk-long dog experiments and 159 rat SA modeling experiments were carried out. These are described in detail below.

In this yr 6 we continued using a systematic approach, aiming for a breakthrough in resuscitation attempts for the presently considered unresuscitable condition of 2 h traumatic ExCA. In yr 1 (1998-99) we established the non-traumatic ExCA model (1,2). In yr 2 (1999-00), we explored pharmacologic adjuncts to hypothermic flush, achieving no breakthrough effect with any of 14 drugs (3). Some benefit came from the antioxidant tempol (4). In yr 3 (2000-01) we pushed profound hypothermic preservation with aortic large-volume saline flush to tympanic temperature (Tty) 5-10°C; we achieved intact survival after a CA of either 60 min or 90 min at 10°C, and inconsistently after CA 120 min (5-7). In yr 4 (2001-02) we documented a 5 min limit to flush delay, pushed the limit of SA to 120 min, and documented problems with coagulopathy when severe tissue trauma was superimposed on our standard exsanguination CA protocol and SA. In yr 4, separate studies also documented the efficacy of SA in a dog model of refractory ventricular fibrillation (VF) CA (17)—setting the stage for the use of this approach even in normovolemic CA victims with sudden cardiac death—those patients that do not respond to standard advanced cardiac life support (ACLS) protocols. In yr 5, using the dog model of ExCA, we built upon the strong foundation of work and carried out 2 important studies. Using re-circulation of the initial flush, we were able to reduce the flush volume from ~400 mL/kg to 50 mL/kg (14). We also successfully tackled the critical challenge of designing an approach that allowed SA to be successfully applied to a 2-h CA with superimposed severe trauma in dogs. We used a contemporary therapy—plasma exchange—to control coagulopathy and facilitate intact survival after 120 min of exsanguination CA with superimposed severe trauma (laparotomy, splenectomy, and thoracotomy) (15). In yr 5, we also began an important additional line of investigation using a rat model of decapitation ischemia–and applying a state-of-the-art proteomic analysis (12,13,18). These studies were designed to define the cascade of global protein degradation that occurs despite profound cooling. Knowing the proteins that are injured during SA will help us define the limits of resuscitability—possibly aiding in the titration of hypothermia (depth, duration), and uncovering the best adjuncts to SA. These studies were carried out under the direct supervision of Larry Jenkins, PhD at the Safar Center.

Germane to work in yr 6, it is recognized that conventional resuscitation after ExCA is often unsuccessful, particularly when prolonged HS produces the arrest. Previously, we reported the success of SA with delayed resuscitation in ExCA. SA of up to 2 h was induced via rapid aortic flush with ice-cold (2°C) saline followed by delayed resuscitation via CPB. This would buy time for transport and/or surgical repair. In the past, we used SA to achieve intact survival of dogs after rapid hemorrhage (over 5 min) to ExCA. We hypothesized that SA would allow survival with good neurological outcome in the setting of prolonged hemorrhage prior to ExCA. Dogs underwent spleen transection and controlled, continuous bleeding until CA. Two min after CA, dogs were randomized into 3 groups (n=7 each): 1) the CPR group resuscitated with conventional CPR and rapid infusion of blood and LR; 2) SA-I, or 3) SA-II Groups, both of which received 20 L of 2°C saline flushed into the aorta. CPR or SA lasted 60 min, and was followed by 2h of CPB and splenectomy. CPR dogs were maintained at 38°C, while SA dogs were controlled at 34°C for either 12h with standard rewarming (SA-I) or 36 h with slow rewarming (SA-II). Outcome was evaluated with Neurological Deficit Scores (NDS) (0%=normal, 100%=brain death) and Overall Performance Category (OPC) (1=normal, 5=death). CA occurred after 124±16 min of hemorrhage. Arterial pH, lactate, and K^+ were remarkably abnormal, and did not differ between groups. In the CPR group, spontaneous circulation could not be restored without CPB; none achieved long-term survival (range: 11.5-16.5 h). In contrast, 12 of 14 SA dogs survived ($p<0.01$ vs the CPR group). The SA-II Group had better NDS than SA-I (1.5 [0-89%] vs 42 [10-92%)], $p=0.04$) and a trend toward better OPC (5 vs 1 dog recovered to normal, $p=0.06$). **Based on this work, we concluded that SA facilitated survival with good neurological outcome in a model of otherwise unresuscitable prolonged hemorrhage with ExCA.** Surprisingly, extending the duration of mild hypothermia followed by slow rewarming after SA was critical to achieving intact neurologic outcome.

Near the end of yr 6, we also began pilot experiments to set the stage for our proposed studies in yr 7, which will tackle the difficult challenge of extending the duration of SA beyond the 2 h barrier. Most promising appears to be the addition of energy substrates such as oxygen and glucose to the flush solution. Approximately 10 successful pilots were carried out that helped define the definitive study of this approach for yr 7.

Also during yr 6, we carried out two series of studies (IIa and IIb) to begin to define mechanism of secondary damage during normothermic and profound hypothermic circulatory arrest (as used in SA). Based on the wealth of molecular agents available in rats (vs dogs), we chose to carry out these mechanistic studies in rats–specifically focusing on global protein degradation (degradomics) in rat brain (hippocampus). Initial work in this area used a decapitation model of complete global brain ischemia (GBI). After decapitation, we isolated the hippocampi, and studied paired hippocampi maintained at target temperature (either 37°C or 10°C—mimicking the temperatures used for either a normothermic insult or SA) for 30 min. Remarkably, during 30 min of ischemia at either normothermia or 10°C, assessment of protein degradation using proteomics (2D gel electrophoresis) revealed minimal degradation (12,13,18). Several possible explanations for these findings included, 1) that reperfusion was critical to the development of protein degradation, 2) that 2D proteomic approaches were not sensitive enough to detect changes in important low copy proteins, or 3) that other targets, such as damage to lipids, DNA, or RNA were more critical to secondary injury after SA. To begin to answer these important questions, we felt it was necessary to develop a complete SA model in the rat—including delayed resuscitation. To achieve delayed resuscitation after these long ischemic times, it was necessary to use a CPB system for use in the rat. We adapted an established miniaturized CPB system for rats that was used by our consultant (David Warner, MD) at Duke University and we successfully established a complete rat model of SA. We have now been able to achieve survival with intact functional outcome in rats after 30 min of SA at 10°C (20). Current studies are optimizing this model and formally evaluating histopathology.

During yr 6 we had 7 publications from work on the SA project, building on a remarkable body of publications from this program (1-23). Fellows working on this project also presented 10 abstracts of this work during yr 6 (abstracts 13-22). Dr. Kochanek, with the research team, also published a chapter in a specific textbook on hypothermia that was presented at an international symposium on hypothermia that was held in Tokyo, Japan (19). Dr. Tisherman was also the lead editor of a textbook on "Therapeutic Hypothermia" (see bibliography).

<u>BODY OF REPORT</u> (yr 6)

Study I) Is SA effective after a prolonged period of HS resulting in ExCA in dogs?

Conventional resuscitation is often unsuccessful after ExCA, particularly when prolonged HS produces the arrest. Previously, we reported the success of SA with delayed resuscitation in ExCA. SA of up to 2 h was induced via rapid aortic flush with ice-cold saline followed by delayed resuscitation via CPB. This would buy time for transport and surgical repair. In the past, we used SA to achieve intact survival of dogs after rapid hemorrhage (over 5 min) to ExCA. The success of SA relies on timely initiation of preservation during CA. It is speculated that different durations of HS before CA may affect efficacy of SA. Rapid Ex does not cause severe tissue acidosis over 5 min while longer duration of HS may exhaust compensatory reserve and build up extremely severe tissue acidosis and tissue injury. Although the CNS is generally damaged minimally during HS, superimposing a transient normothermic CA and a prolonged profound hypothermic CA, i.e. SA, upon prolonged HS may importantly complicate the ability to preserve and resuscitation. **Thus we designed a clinically model particularly relevant to military and civilian trauma that is characterized by a controlled continuous bleeding, trauma (laparotomy and spleen transaction), limited fluid resuscitation, and resultant CA. Allowing volume depletion and circulatory decompensation to finally cause CA was intended to create a setting that is non-salvageable with conventional CPR.**

The dog model (Fig 1) included 3 phases: 1) HS and CA phase: bleeding was continuous until CA; 2) CPR/SA phase: 2 min after CA, dogs were resuscitated with either conventional CPR or SA; and 3) delayed resuscitation phase, including 2 h CPB, and up to 72-96 h intensive care. Dogs were assigned to 1 of 3 groups 2 min after CA: 1) the CPR group was resuscitated with conventional CPR; 2) the SA-I group was resuscitated with arterial flush of 20 L ice-cold saline, followed by delayed resuscitation with CPB, 12 h of post-ischemia mild hypothermia, and was sacrificed at 72 h; and 3) the SA-II group was identical to the SA-I group except that 1) the duration of post-ischemic hypothermia was 36 h, 2) mild hypothermia was reversed by slower rewarming, and 3) these dogs were sacrificed at 96 h.

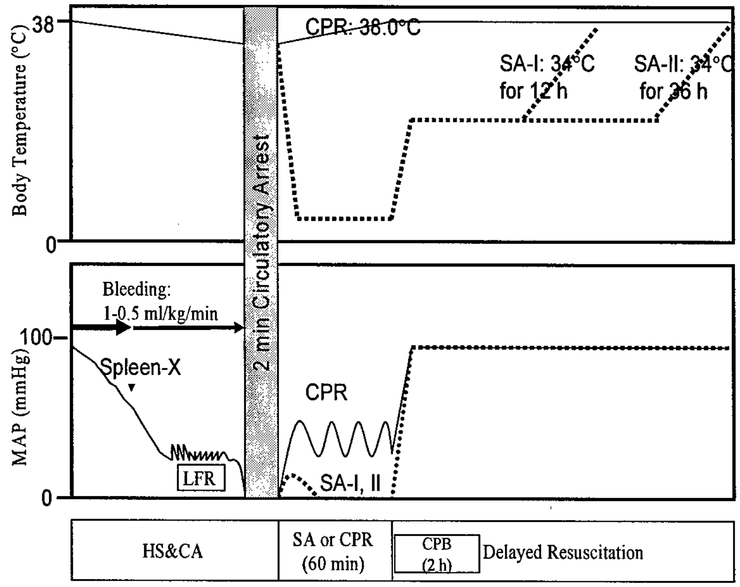


Figure 1. Experimental protocol for testing the efficacy of SA after prolonged HS in dogs. SA-I = conventional SA; SA-II = conventional SA followed by 48 h of mild hypothermia during ICU care.

At HS 0 min, venous blood was withdrawn via the right femoral vein catheter at a rate of 1 ml/kg/min over 40 min. Out-going blood was spiked with 0.125 ml/kg/min citrate delivered via a PE 60 catheter that ran inside the femoral vein catheter lumen. From HS 40 min, blood withdrawal rate was set at 0.5 ml/kg/min. At HS 40 min, the spleen was transected. When MAP was lower than 30 mmHg, limited fluid resuscitation was started with infusion of 100 ml of LR over 2 min. The maximum amount of LR was 500 ml. ExCA was defined as: 1) MAP <10 mmHg, and severe bradycardia (<20 bpm) or, 2) asystole or VF confirmed by EKG and arterial blood pressure tracing.

The original design included only the CPR and SA-I Groups. The SA-II Group was added into the study for randomization after 10 dogs in the CPR and SA-I Groups were completed. Two min after CA, dogs were randomized into the CPR or SA Groups. In the CPR Group, the conventional ACLS was started. Briefly, chest compression with a thumper was started at 60 beats per min, and the compressing distance was adjusted in an attempt to generate a systolic blood pressure of 100 mmHg. Ventilation with 100% O_2 was provided at 12 breaths per min, and the airway pressure was set at 40 cm H_2O. Epinephrine 0.01 mg/kg was given every 5 min (maximum 5 times). After epinephrine administration, defibrillation with 150 J was given when EKG showed VF; increment of 50 J was given after 2 unsuccessful defibrillations. Sodium bicarbonate and $CaCl_2$ were given if BE<-6 mmoml/L and

Table 1. Physiological parameters during CA preceded by prolonged HS

	CPR Group	SA-I Group	SA-II Group
Hemorrhage Time (min)	124.4±10.5	118.4±19.7	126.4±19.8
pH	6.88±0.24	6.99±0.16	6.93±0.12
PCO_2 (mmHg)	86±39	58±30	73±28
PO_2 (mmHg)	58±8	85±31	79±24
BE (mmol/L)	-16.6±2	-16.4±1.3	-15.5±1.8
K+ (mmol/L)	7.6±1.2	6.8±1.3	7.3±0.9
Glucose (mg/dl)	449±150	563±110	522±207
Lactate (mmol/L)	15.1±1.6	14.6±2.8	14.1±2.3
BUN (mg/dl)	23.4±6.1	27.6±6.5	25.6±8.1
Hematocrit (%)	16.2±2.2	18.1±2.3	19.6±1.8

Ca^{2+} <1 mmol/L. At CPR 0 min, LR 1 L was infused over 10 min, and shed blood 30 ml/kg was given over 5 min. Additional LR (250 ml/15 min for 3 times) was given afterwards. In the SA Groups, aortic flush of 20 L of 2°C saline via CPB cannula was initiated at a rate of 1.6 L/min via a roller pump (Ardiem, Indiana, PA). The dog was then covered with ice after the flush.

Sixty min after the onset of aortic flush or CPR, CPB was started. At RT 0 min, splenectomy was performed. The abdominal wounds were closed in 3 layers. The temperature in the CPR Group was kept at 38°C, while it was rewarmed to 34°C in about 1 h in the SA Groups. Defibrillation was attempted when splenectomy was completed in the CPR Group and the core temperature reached 32°C in the SA Groups. ICU care was provided for 24 h in the CPR and SA-I Groups, and for 48 h in the SA-II Group. In the CPR Group, the body temperature was kept at 37.5-38.5°C. In the SA-I Group, the body temperature was kept at 34°C until RT 12 h, which was followed by standard rewarming (1°C/h) to 37.5°C. In the SA-II Group, mild systemic hypothermia (34°C) was maintained for 36 h, followed by a slower rewarming (0.3°C/h) to 37°C than in the SA-I Group. Outcome was evaluated according to OPC. Neurologic function was evaluated as NDS. In selected dogs, paraformaldehyde fixed brain sections were stained with H&E or Fluoro-Jade B and analyzed by a neuropathologist blinded to treatment.

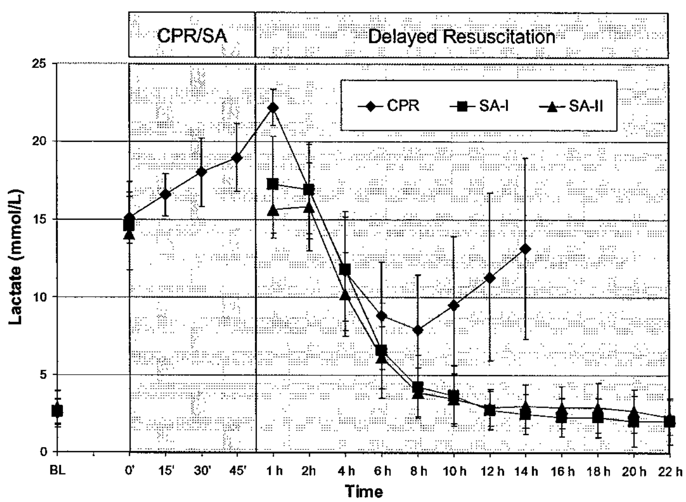


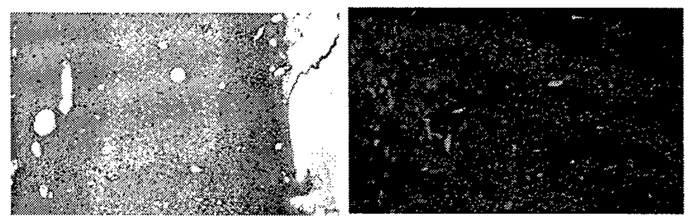
Figure 2. Blood lactate levels after ExCA preceded by prolonged HS. Blood lactate was markedly elevated in all 3 groups at the time of arrest, but recovered in the SA group. In contrast, lactate never completely recovered after conventional CPR and there was a secondary rise that accompanied the development of multiple organ failure and death in the CPR group.

The averages of hemorrhage time before CA were 124±16 min in all groups, and there was no difference between groups (Table 1). Arterial pH, PCO_2, PO_2, BE, K^+, lactate, and glucose values at 1 min after CA were markedly abnormal, but similar in 3 groups (Table 1).

During chest compression, MAP was kept >50-60 mmHg over 1 h. However, return of spontaneous circulation was not achieved by CPR/ACLS in any dog in spite of vigorous resuscitation efforts with chest compression, ventilation, medications (epinephrine, calcium chloride, bicarbonate), fluid, blood, and defibrillation.

Return of spontaneous circulation was achieved in all dogs 15±16 min after initiation of CPB with 157±181 J defibrillation. However, substantial fluid losses developed during recovery phase from the rectum, orogastric tube, and abdominal drainage catheter (all p<0.01 vs the SA Groups) and multiple organ failure ensued. The lactate levels decreased transiently, but increased sharply again until death ($p<0.01$, vs the SA Groups) (Fig 2).

At the end of aortic flush with 20 L of ice-cold saline, Tty was decreased to similar levels in both SA Groups. It remained almost unchanged during preservation. Dogs were rewarmed to 34°C within 1 h by CPB. When the core temperature reached 32°C, defibrillation successfully restored circulation in all dogs at 30±12min in the SA-I Group, and 32±23 min in the SA-II Group (NS).



A: Frontal lobe, H&E　　　B: Cerebellum, Fluoro-Jade B

Figure 3. Brain histology in SA-1. See text for details.

Preliminary Brain Histology: In 2 SA-I dogs that developed generalized seizures after 48 h, extensive laminar necrosis was seen in the cortex (Fig 3A) and synaptic damage was seen throughout the cerebellum (Fig 3B). A complete histopathological analysis of 19 brain regions in each dog is ongoing.

Outcome: All CPR dogs died with a median survival time of 14.7 h (range 11.5-16.5h) ($p<0.01$, vs the SA Groups). In contrast, all SA-I dogs survived to 72 h except 1 that died at RT 29.5 h ($p<0.01$ vs the CPR Group). Similarly, in the SA-II Group, 6/7 survived to 96 h ($p<0.01$, vs the CPR Group). Of all survivors, 2 SA-I dogs developed generalized seizures 48 h after extubation. One had seizures shortly after weaning of sedation at RT 24 h. At 72 h, there was only one dog that recovered to normal. In contrast, none of SA-II survivors exhibited seizures and 5 of 6 recovered to normal at 96 h ($p=0.06$, vs the SA-I Group) (Table 3). **The final NDS at the 96 h in the SA-II Group was significantly better than that in the SA-I Group at RT 72 h ($p=0.04$).**

In this study we established an ExCA model that is unsalvageable using contemporary conventional resuscitation. At the time of CA, ~60-90% of the blood volume was removed. Arterial blood gases taken at the beginning of CA showed severe acidosis (pH<7.0), hyperkalemia, and hyperlactemia. As expected, none could be resuscitated despite an aggressive contemporary resuscitation. Although all of the dogs could be resuscitated with CPB, all subsequently died of severe multiple organ failure, including renal failure, extensive gastrointestinal mucosa necrosis and sloughing, and cardiovascular dysfunction. This pattern is what was anticipated in the setting of conventional resuscitation after prolonged HS and CA. In contrast, SA with delayed resuscitation significantly improved survival. Twelve of 14 dogs survived without apparent extra-cerebral organ injuries. However, dogs in the SA-I Group had severe neurological injuries. Three dogs had generalized seizures after weaning from mechanical ventilation and sedation. Initially, two of them regained consciousness but became comatose following episodes of seizures that occurred after 48 h. This is a unique pattern that we had not previously encountered in the long history of investigation of rapid ExCA models. Extensive laminar necrosis was found in the cortexes, and synaptic injuries were found in the cerebellum. This is very different from the histopathology observed after rapid ExCA in which almost no brain damage was seen after 60 min of SA. The SA and delayed resuscitation protocols were almost identical between these two studies. Apparently, the pre-existing prolonged HS substantially decreased the efficacy of SA.

The mechanism of delayed neurological deterioration after prolonged HS and prolonged hypothermic CA that was seen with our standard rewarming protocol may be related to delayed cytotoxic brain edema that peaks around 48 h after reperfusion. Different from rapid ExCA, the blood glucose level at time of CA was >500 mg in most dogs. Based on a similar histological pattern coupled with previous reports of hyperglycemia-associated brain injury, it is speculated that the high blood glucose levels may contribute to the delayed neurological deterioration. Alternatively, rapid rewarming of the traumatically injured brain can markedly exacerbate injury. **Regardless of what exact mechanisms may be responsible, hypothermia has been found to be very effective in protecting against ischemic brain injuries**. Remarkably similar to our finding in profound hypothermic CA, Gunn et al (1997) documented that delayed brain edema that peaked at 48 h after 30 min cerebral ischemia in fetal lamb was abolished by prolonged (72 h) hypothermia. Shorter term (48 h) cooling was associated with rebound of epilepitiform activity. Similarly, Colbourne et al (1994) found that to salvage CA1 neurons after 5 min of ischemia in gerbils, hypothermia (32°C), induced 1 h after ischemia, needed to be maintained for 24 h rather than 12 h. **In our study, when the duration of hypothermia was extended to 36 h, followed by slow rewarming (0.3°C/h), 6 of 7 dogs regained consciousness, and seizures were not seen, even when observation was extended to 96 h.**

Table 2. Survival Rates and OPC in Survivors of SA preceded by prolonged HS

	CPR N=7	SA-I N=7	SA-II N=7
OPC 1		•	•••••[#]
OPC 2		•	
OPC 3		•••	
OPC 4		•	•
OPC 5			
Death <72 h	•••••••[##]	•	•

[#]p=0.06, vs 1/6 in the SA-I Group;## p=0.001 for survival for SA-I or SA-II.

We were surprised by the dramatic mild effects of post-ischemic mild hypothermia in our clinically-relevant model. The success of resuscitation of CA after prolonged HS as demonstrated in this study may have important implications. First, it is suggested that the potential application of SA is not limited by the duration of pre-existing HS. This may facilitate application of a novel therapeutic approach for otherwise lethal ExCA. Second, SA may provide an effective treatment for a specific situation in modern urban wars as the one in Somalia in 1993 in which several US soldiers were wounded and pinned down for >14 h until they could be evacuated (Mabry et al., 2000). Such a prolonged uncontrolled HS poses as a new challenge to military medical care. As suggested in our study, SA is much more reliable in preserving tissue viability during CA than conventional CPR. It is plausible that SA may allow intact survival in the setting of prolonged HS. Third, the surprising benefits of prolonged post-ischemic mild hypothermia and slow rewarming may also provide neuroprotective effects after deep hypothermic CA (DHCA) as used in cardiac or neurological surgeries. This could have potential value, for example, in cases where neurologic injury is suspected after open-heart surgery. Currently, rapid rewarming is commonly practiced after DHCA. In conclusion, SA facilitated survival with good neurological outcome in a model of otherwise unresuscitable prolonged hemorrhage with ExCA. Surprisingly, extending the duration of mild hypothermia and reversing it at a slower rate after SA was critical to achieving intact neurologic outcome.

Study II) Mechanistic studies of SA in rats
(IIa) Proteomic assessment of global protein degradation (degradomics) in SA and (IIb) Development of a complete rat model of SA

(IIa) Proteomic/degradomics assessment of protein degradation in SA
In FY6, we first chose to build on our work assessing protein degradation in rat brain hippocampus after prolonged periods of complete global brain ischemia (GBI) using two dimensional (2D) gel electrophoresis without recirculation. Complete GBI without recirculation is an ideal model to evaluate homogeneous CNS changes since the ultrastructural responses vary little among different brain regions or cell types. We examined hippocampal proteomic changes after 30 min of GBI at normothermia vs hypothermia to identify any predictive patterns of protein degradation or post-translation modification. Hypothermia at 10°C is optimal in our dog SA model—and was thus selected for use in these studies. In yr 5, using large format 2D gel, we noted surprisingly minimal differences in protein degradation with comparing 30 min of GBI at either 38 or 10°C (). However, weaker solubilization buffers are required for 2D gel vs 1D gel analysis. We sought to further evaluate global protein degradation during prolonged complete GBI with and without hypothermia using more powerful solubilization buffers and 1D gel analysis. Our <u>hypothesis</u> was that protein degradation is minimal during prolonged normothermic or hypothermic ischemia in rat brain.

Using a decapitation complete GBI model in Sprague-Dawley rats (n=6 per group), both hippocampi were rapidly dissected and randomized to 30 min of complete ischemia at either 38 or 10°C. A third group of hippocampi (no ischemia) served as controls. Separation of proteins from hippocampal lysates by molecular weight was accomplished with medium format (16X18 cm) SDS-PAGE. Paired samples were run in triplicate on the same gel to reduce variability, stained with Sypro Ruby, imaged and quantified.

No differences in protein levels were found between either normothermic or hypothermic ischemia groups—or controls—without reperfusion (Fig 4).

We observed little degradation of the rat hippocampal proteome during prolonged normothermic or hypothermic complete ischemia without reperfusion. These data confirm (using a separate method) and extend our prior work with 2D gel analysis. In that multiple studies have shown important neuroprotection by profound hypothermia during complete GBI, we are currently

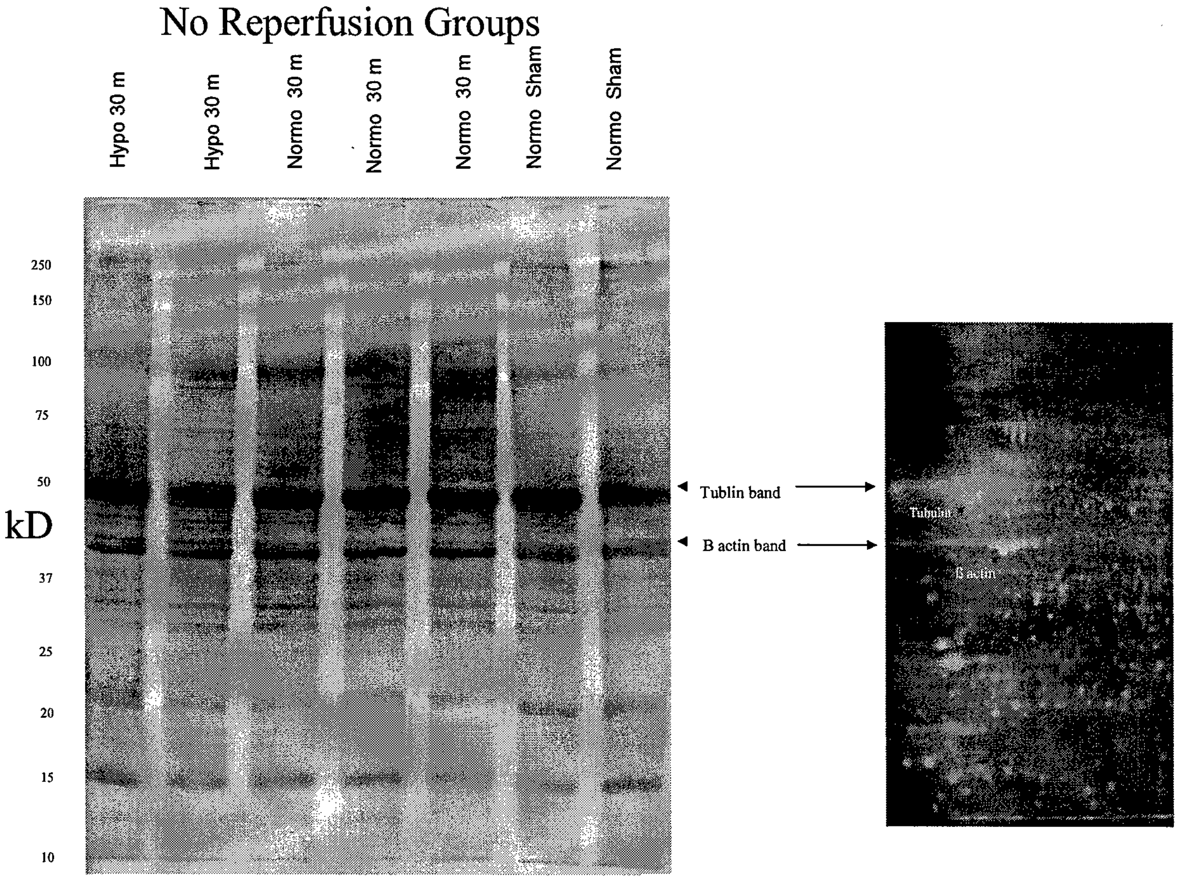


Figure 4. Protein degradation in rat hippocampus after 30 min complete GBI without reperfusion. Left panel shows 1D analysis of each group in triplicate stained with sypro ruby. Right panel shows Sypro Ruby stained 2D gel showing that each of the 1D bands represent a large number of proteins, for example in the high copy tubulin and β-Actin identified.

examining the effect of reperfusion on hippocampal protein degradation after prolonged normothemic cardiac arrest and SA using our newly established complete rat SA model (described below).

(IIb) Development of a complete rat model of SA

SA with delayed resuscitation is a novel approach that we have developed for resuscitation of ExCA victims. SA utilizes cold aortic flush to induce a state of hypothermic preservation, followed by resuscitation with CPB. In prior reports, we have used a dog model to study long-term outcome and maximize clinical relevance. However, because of the limited availability of molecular tools in dogs, development of a rat model of SA would enable further investigation of the mechanisms underlying secondary neuronal injury during SA and delayed resuscitation. We tested two hypotheses; first, that SA would be achievable in a rat model, and second, that plasmalyte would be a more favorable aortic flush solution than normal saline.

HS was induced with rapid exsanguination of 12.5 ml of blood over 5 min, followed by KCl-induced CA. After 2 min of no-flow, cooling to 10°C (target temperature) was initiated with ice-cold flush (30 ml/min flow rate, total volume 500 ml), and topical cooling. After 30 min of DHCA, reperfusion and re-warming were achieved with a miniaturized CPB system over 60 min. In all rats, arterial blood gases, Hct, electrolytes, glucose, lactate, BUN and Osm were serially monitored. Rats were extubated 2 h later and body temperature controlled at 34°C with telemetry. Survival to 24 h was the primary outcome variable. Outcome was also assessed at 7 d using an OPC and a NDS modified for rats.

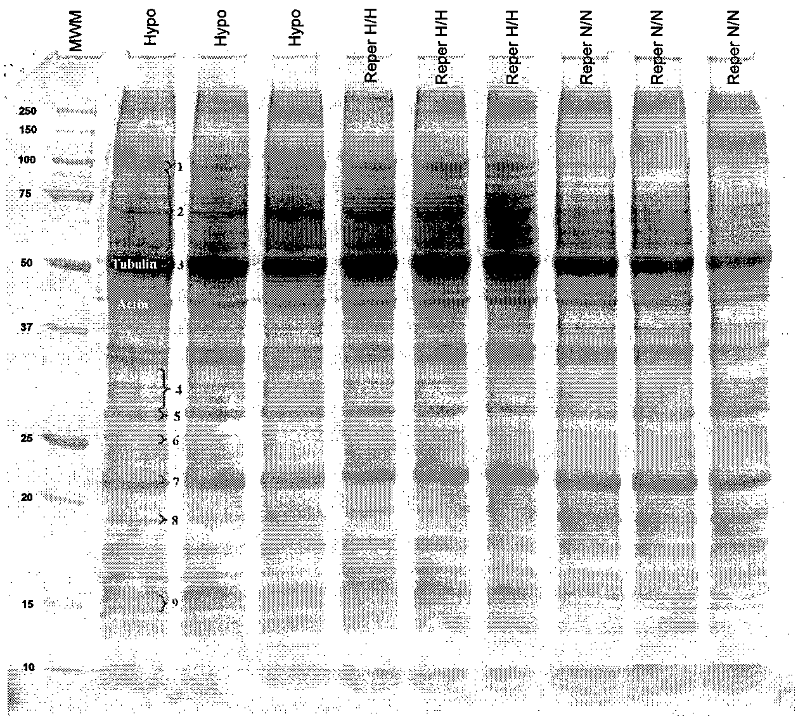


Figure 5. Preliminary data using 1D gel electrophoresis showing the effect of reperfusion on protein degradation after 30 min of normothermic CA vs 30 min of SA (at 10°C) in rat hippocampus. From left to right the vertical lanes show SA without reperfusion (Hypo, 3 lanes), SA with reperfusion (Reper H/H, 3 lanes) and normothermic CA with reperfusion (Reper N/N, 3 lanes). Reperfusion was achieved for 30 min using CPB. As seen in Fig 4, prolonged ischemia without reperfusion produced not obvious protein degradation. In contrast, reperfusion after normothermic CA resulted in obvious loss of high molecular weight proteins (in areas 1-2) and increases in lower molecular weight proteins (such as in areas 7 and 9). This suggests a critical role of reperfusion to protein degradation in SA.

Eighteen rats were used; 4 rats died from technical reasons. Flush with ice-cold normal saline or plasmalyte rapidly decreased the temperature to 10°C. 4/7 rats survived to 24 h in both normal saline and plasmalyte groups. Favorable outcome (NDS <10%) at 7 d was achieved in 2/7 rats in the normal saline and 4/7 in plasmalyte group.

We have successfully established an SA model in rats that includes 30 min of profound hypothermia and resuscitation using miniaturized CPB. To our knowledge, this is the first successful description of either SA or DHCA in a rat. The model is technically demanding but can achieve intact long-term survival. Our preliminary results suggest a favorable outcome with Plasmalyte vs normal saline as the flush solution. Successful establishment of this model of SA in rats should facilitate application of molecular tools for studying the effects of both SA and DHCA and reperfusion on mechanisms of neuronal death. Our preliminary data using this model suggest that reperfusion is critical to protein degradation in SA (Fig 5). As discussed previously in this report, this model may also serve to screen approaches to neuronal preservation in both SA and DHCA with obvious relevance to cardiac surgery and transplantation medicine.

Other accomplishments of the SA program during yr 6

Devices developments: In yr 5, we established a steering committee with Dr. Lyn Yaffe as administrative chairman, to coordinate laboratory results from this Army project, developments of methods and devices, and planning clinical trials of mild hypothermia for traumatic HS and profound hypothermic aortic flush SA for exsanguination CA. This steering committee includes the Pittsburgh team (Kochanek, et al, for SA, and Tisherman, et al, for HS), Yaffe (smart catheter project), McMurray, Ardiem (portable cooler project), and Dr. Tisherman, et al, for planning clinical trials. That steering committee continued to meet via telecom throughout the year in conference calls at a minimum of every other week, and in person with Dr. Yaffe visiting Pittsburgh about once per month. The project of Dr. Yaffe includes Dr. Klain, and as advisors, Drs. Kochanek and Tisherman, and Mr. S. W. Stezoski. These conference calls have proven invaluable to our team.

Several dog experiments were also used for testing of adjunctive methods and devices, before euthanasia, to save extra dog lives. These efforts (which are approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh) continue to lead to prototypes of aortic balloon catheters that are now being improved. Ardiem delivered its first prototype of a portable cooler to us in Sept. 2003. That cooling device was used to induce SA in each of the dog experiments performed in the prolonged HS study described above.

Miscellaneous: For clinical trials, Dr. *Tisherman* continues to communicate with 6 potentially participating major trauma hospital groups.

Xianren Wu, MD continued to serve as senior fellow on the dog SA project in 2004 under the mentorship of Drs. Kochanek and Tisherman. He has remarkable experience, particularly for a fellow, in the field of HS including work in dog, pig, and rat models. His productivity regarding publications has been exceptional.

Mandeep Chadha, MD a fellow working under the direction of Drs. Jenkins and Kochanek is using proteomics to study protein degradation and modification during complete global cerebral ischemia with profound hypothermia. Dr. Chadha is a critical care medicine fellow who is funded separately by a T-32 entitled Pediatric Neurointensive Care and Resuscitation Research from the NICHD/NIH on which Dr. Kochanek is PI and Dr. Jenkins is a trainer. This project on proteomics in SA has broad implications across the field of resuscitation and is an outstanding opportunity for fellowship training. Dr. Chadha has presented numerous abstracts of his novel work in proteomics and an initial full manuscript is in preparation.

Tomas Drabek, MD joined our group this year as the fellow in charge of development of the rat SA model. Dr. Drabek is a practicing cardiac anesthesiologist in Prague, Czech Republic, and his experience in that regard is perfect to expand the relevance of our rat SA model to the study of DHCA in cardiac surgery. He has submitted an initial abstract of the complete rat SA model to the 2004 meeting of the Society of Critical Care Medicine.

During yr 5, our group gave over 13 presentations on our work on the SA project. This included abstracts presented by Drs. Wu, Chadha, Drabek, and invited lectures by Drs. Kochanek, Tisherman, and Jenkins.

Second Annual Safar Symposium at the University of Pittsburgh School of Medicine: On October 30, 2003, the second *Annual Safar Symposium* was held at the University of Pittsburgh School of Medicine. This event was attended by ~150 clinicians and scientists. The morning session focused on "Breakthroughs in Resuscitation" and again focused on resuscitative hypothermia. The afternoon focused on the use of simulation in resuscitation research. The symposium featured prominent national and international speakers and was supported in part by this grant. The program is attached in the appendix. We again thank the US Army for supporting this symposium in honor of Dr. Safar.

Books and monographs: Dr. Tisherman was the lead editor of a textbook on hypothermia in acute medicine entitled 'Therapeutic Hypothermia." That book is in press and will be published by Kluwer. In addition, Dr. Kochanek was the lead editor of a supplement to the February 2004 issue of the journal Critical Care Medicine that was assembled as a special Festschrift in honor of Dr. Peter Safar. That supplement highlighted a number of aspects of this SA project. We are indebted to the US Army (TATRC) for supporting this publication.

KEY RESEARCH ACCOMPLISHMENTS

Accomplishments for funding yr 6

Study I: We showed that SA is still effective even when a remarkably prolonged 1.5-2.5 h period of HS precedes the CA. This has important implications for potential clinical use in the field hospital, trauma bay, or armored far-forward field hospital. This expands the potential target of casualties for which SA might be applicable.

Study IIa: Our initial studies on the application of proteomics to SA suggest that protein degradation during the hypothermic insult is minimal. This is an unexpected finding and suggests that reperfusion after the insult is likely the time of significant protein degradation providing an expanded therapeutic window to intervene and better preserve protein function with novel therapies.

Study IIb: Our group has been able to successfully develop a complete rat model of SA, including application of miniaturized CPB to resuscitate after 30 min of DHCA. To our knowledge, these are the first successful studies of DHCA in the rat. These studies should greatly facilitate the study of the effect of reperfusion after SA, and provide the ability for us to screen novel therapies.

REPORTABLE OUTCOMES

Specific reportable outcomes for yr 6 are defined in the report and identified with an asterisk (*) in the reference list—including publications and presentations.

CONCLUSIONS

Work in yr 6 of this project has continued to expand the scope of the potential application of SA for combat and civilian casualties. Specifically in yr 6, we have demonstrated that SA is still effective even when a remarkably prolonged 1.5-2.5 h period of HS precedes the CA. We also carried out parallel mechanistic studies in a rat model of decapitation global brain ischemia to identify key assess protein degradation using novel proteomics methods. Finally, in yr 6, we were able to establish a complete rat model of SA. Future studies will further refine SA in our dog and rat models and also mild hypothermia to optimize "Emergency Hypothermia" in lethal HS while we begin to plan and implement feasibility clinical trials.

References and Appendices

This reference and appendix list includes all items generated by the SA project during years 1-6. *Denotes items generated during funding yr 6. Reprints of these specific items from yr 6 are attached in the appendix, which has been sent under separate cover, since some of these items (books, etc) are not available on PDF. Reprints from prior years are available upon request.

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